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Effect of Growth Rate on the Production of L-Proline in the Fed-batch Culture of *Corynebacterium acetoacidophilum*

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Abstract *Corynebacterium acetoacidophilum* RYU3161 was cultivated in a L-histidine-limited fed-batch culture. To investigate the effect of cell growth on the L-proline production, 5 L fed-batch culture was performed using an exponential feeding rate to obtain the specific growth rates (μ) of 0.04, 0.06, 0.08, and 0.1 h^{-1}. The results show that the highest production of L-proline was obtained at μ = 0.04 h^{-1}. The specific L-proline production rate (Q_P) increased proportionally as a function of the specific growth rate, but decreased after it revealed the maximum value at μ = 0.08 h^{-1}. Thus, the highest productivity of L-proline was 1.66 g L^{-1} h^{-1} at μ = 0.08 h^{-1}. The results show that the production of L-proline in *C. acetoacidophilum* RYU3161 has mixed growth-associated characteristics.

Keywords: *Corynebacterium acetoacidophilum*, L-proline, fed-batch culture, specific growth rate

L-Proline (2-pyrrolidinecarboxyic acid) is a pharmaceutically important non-essential amino acid that has been used as an additive for drug and feed [1]. It is non-polar, hydrophobic and a cyclic aliphatic amino acids in the glutamate family. The biosynthetic pathway of L-proline has been studied in *Escherichia coli* and other bacteria [2-4]. It was first demonstrated by Vogel and Davis that L-proline is synthesized from the glutamic acid by three enzymatic reactions and one spontaneous cyclization in the mutant of *E. coli* [2].

Many efforts have been performed for the fermentative production of L-proline. Most of L-proline producers were bacteria such as the *Corynebacterium* or *Brevibacterium* species. These L-proline producers are composed of different species with different properties including: the L-isoleucine auxotroph of *B. flavum* [3,5], the nucleic acid base auxotroph of *C. glutamicum* [6], and the L-phenylalanine and tyrosine auxotroph of *C. mealssecola* [7].

In a previous study, we developed a high yield strain for L-proline production by mutation methods such as NTG and UV irradiation. *C. acetoacidophilum* RYU3161 was isolated as a high yield strain for L-proline and showed characteristics that were resistant to sulfaguanidine and proline analogs, furthermore was found to be L-histidine auxotroph [8]. In this study, a fed-batch culture of the mutant was investigated for the improvement of L-proline production. In general, the most commonly used fed-batch operational strategies are a constant feeding rate, an exponential feeding, a substrate concentration control, and respiratory quotient (RQ) control strategies. In this study, the effect of cell growth on the L-proline production was investigated by using the exponential feeding strategy during the fed-batch culture.

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Corynebacterium acetoacidophilum RYU3161 (KCTC 0616BP) was used in this study. The strain was a L-histidine auxotroph, and had characteristics that were resistant to sulfaguanidine and proline analogs (DHP, AZC, TAC) [8]. For the seed culture, YPD medium (2% glucose, 1% yeast extract, 2% Bacto-peptone) was used. A single colony on the YPD agar plate was inoculated into 30 mL of YPD medium and incubated at 30°C for 24 h. For further activation, 10 mL of the seed culture was transferred into two 500-mL Erlenmeyer flasks containing 100 mL of the same medium, and incubated at 30°C for 12 h. The batch culture medium was as follows: glucose 60 g/L, yeast extract 7 g/L, KH_2PO_4 3 g/L, $(NH_4)_2SO_4$ 30 g/L, biotin 0.3 mg/L and thiamin 1 mg/L, and trace solution 1 mL. The composition of the trace solution was as follows: $FeSO_4$ 3 g, $MnSO_4$ 3 g, and $ZnSO_4$ 2 g in 100 mL of 0.1 N HCl. The feed medium was formulated as follows: glucose 400~900 g/L (In previous study, glucose consumption rate increased as a function of specific growth rate (unpublished). Therefore, as specific growth rate increased, glucose concentration was increased to avoid the glucose-limited condition [9], $MgSO_4 \cdot 7H_2O$ 5 g/L, K_2HPO_4 10 g/L, Na_2HPO_4 15 g/L, yeast nitrogen base without amino acids 10 g/L, L-histidine 1.5 g/L, $(NH_4)_2SO_4$ 40 g/L, and trace solution 10 mL. The seed culture that was grown on the YPD medium was inoculated with 10% (v/v) and put into a 5 L jar fermentor (Korea Fermentor Co., Korea) containing 2 L of a batch culture media. When the optical density was reached at about 20 OD, the feeding media was

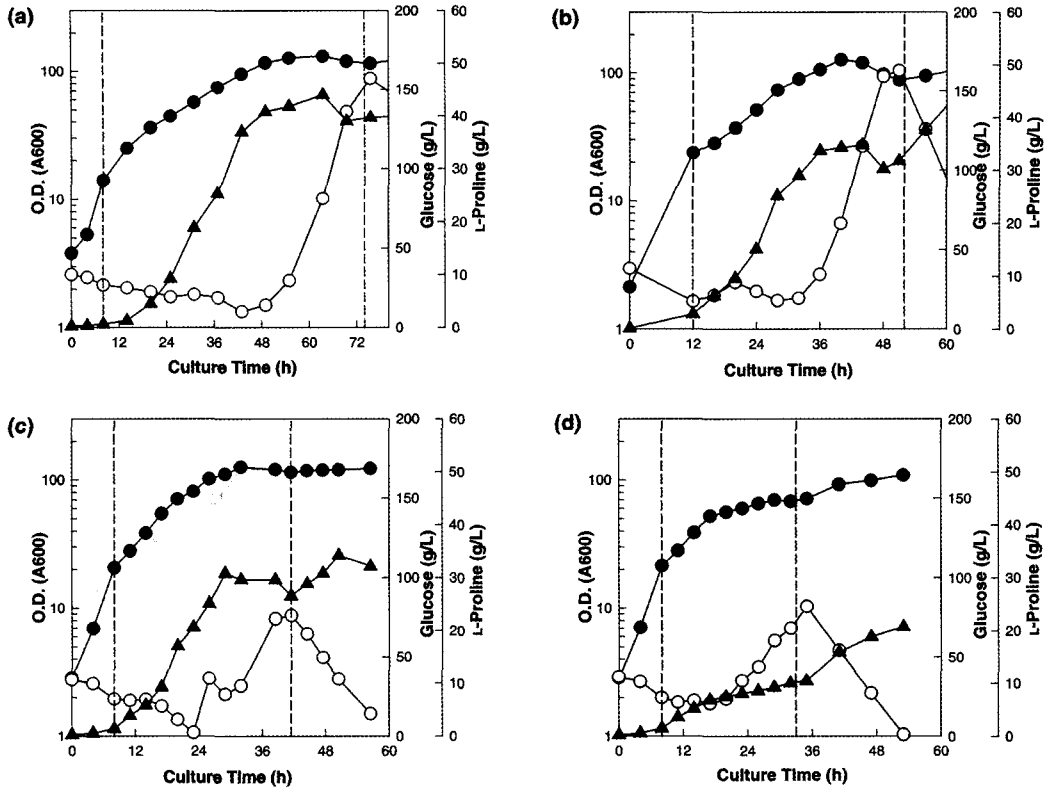

Fig. 1. Profiles of the experimental biomass, residual glucose, and L-proline production in a fed-batch culture using an exponential feeding rate for constant specific growth rate (μ). (a): μ=0.04 h^{-1}, (b): μ=0.06 h^{-1}, (c): μ=0.08 h^{-1}, (d): μ=0.1 h^{-1}. Dotted lines show the period between the start of the exponential feeding and its end. Symbol: ● optical density, ○ residual glucose concentration, ▲ L-proline concentration.

continuously fed to the fermentor through a peristaltic pump (Masterflux co., USA). In order to control the cell growth at a particular specific growth rate, the exponential feeding method was used, and the medium feed rates were automatically calculated by computer using the following equation. Where F is the

$$F = \frac{\mu X_0 V_0 \exp(\mu t)}{Y_{X/S} S_F} \quad (1)$$

volumetric medium feed rate (Lh^{-1}), μ is the desired specific growth rate (h^{-1}), X_0 and V_0 are the cell concentration (g/L) and the culture volume (L) at the beginning of the fed-batch, and $Y_{X/S}$, and S_F represent the biomass yield to L-histidine, and the L-histidine concentration (g/L) in the fermentor and the feed medium, respectively.

The cell growth was monitored by measuring the optical density (OD) at 600 nm (UVICON930, Switzerland). Dry cell weight was estimated by a pre-determined conversion factor of 0.24 g dry cell weight/L/OD. To determine the residual glucose concentration, 1 mL of culture broth was centrifuged and the glucose concentration of the supernatant was measured by a glucose and L-lactate analyzer (YSI Model 2000, Yellow Springs, Ohio, USA). L-Proline concentration was determined by using the ninhydrin reaction method [10].

When microbial products are growth-associated, their specific growth rate (μ) is a key factor in the optimization of microbial culture. Microbial cultures with a controlling specific growth rate have been performed for the improvement of the productivity of microbial products [11-14]. Therefore, in order to investigate the effect of cell growth on L-proline production and to find the optimal specific growth rate for the production of L-proline, a fed-batch culture of *C. acetoacidophilum* RYU3161 was performed using the exponential feeding method. This feeding method is a feed-forward control method in which the feeding rate is predetermined. It was based on equation (1). As a growth factor for the control of *C. acetoacidophilum* growth rate, L-histidine was used, because the high production of L-proline was obtained under the L-histidine-limited condition (unpublished). The biomass yield to L-histidine ($Y_{X/S}$: 57.1 $g_{cell}/g_{L\text{-histidine}}$) required in equation (1) was obtained through the study of chemostat culture of *C. acetoacidophilum* (data not shown). Exponential feeding was initiated at the point of initial L-histidine depletion. This was when the optical density was reached at about 20 OD. Fig. 1 shows the time profiles of the fed-batch culture with exponential feeding method to maintain the specific growth rate (μ=0.04, 0.06, 0.08, and 0.1 h^{-1}), respectively. In all of the cultures except for μ=0.1 h^{-1}, specific growth rate effectively remained con-

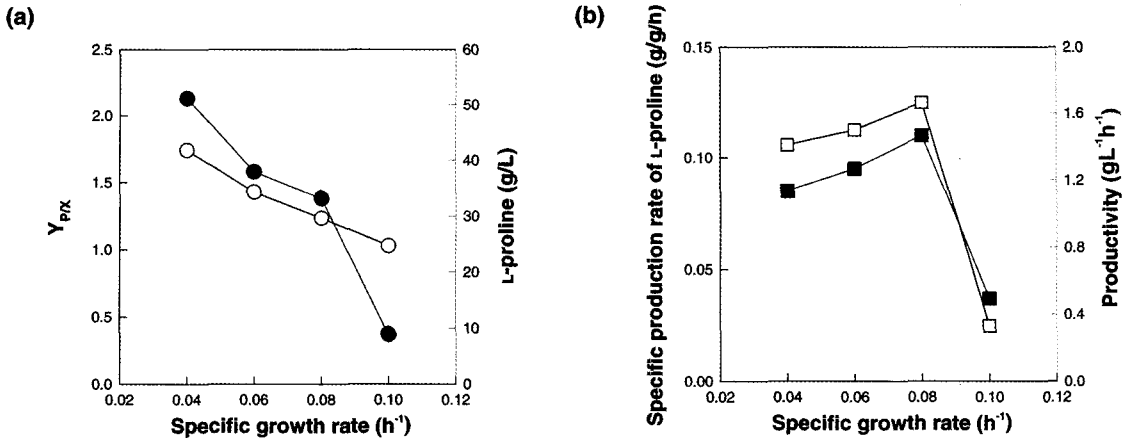

Fig. 2. Changes of the main parameters obtained in the fed-batch cultures with the exponential increased feeding rate calculated to maintain various specific growth rates. Symbol: ● $Y_{P/X}$ ($g_{L\text{-proline}}/g_{cell}$): yield for L-proline produced per biomass formed, ○ L-proline (g/L): mass of produced L-proline until biomass reached 30 g/L of dry cell weight, ■ Q_P ($g_{L\text{-proline}}/g_{cell}/h$): specific production rate of L-proline, □ productivity (g L^{-1} h^{-1}).

stant until the optical density reached approximately 100 OD. It was observed that glucose had accumulated at a high cell density in all of the cultures (Fig. 1). It was difficult to keep the specific growth rate constant with the high cell density in all of the culture that had a high growth rate (μ=0.1 h^{-1}), which may have resulted from the oxygen-limited growth of *C. acetoacidophilum* and the accumulation of glucose. The production of L-proline was associated with cell growth regardless of how the μ was maintained at the beginning of the all fed-batch cultures. However, there were variations in the profiles depending on the μ value (Fig. 1). For μ=0.04 h^{-1}, the L-proline production became stable at 42 g/L, after 40 h of the fed-batch operation (Fig. 1(a)). For μ=0.06 and 0.08 h^{-1}, L-proline production became stable at 33 g/L after 24 h and 30 g/L at 20 h, respectively (Figs. 1(b) and 1(c)). For μ=0.1 h^{-1}, L-proline production showed the lowest value, at around 10 g/L (Fig. 1(d)).

To investigate the effect of the specific growth rate on the production of L-proline, the specific growth rate was correlated with the L-proline/biomass yield ($Y_{P/X}$) and the L-proline production, the specific L-proline production rate (Q_P) and the productivity effectively remained constant in all of the cultures during specific growth rate. $Y_{P/X}$ decreased as a function of specific growth rate, and showed the highest values (2.13 $g_{L\text{-proline}}/g_{cell}$) at the lowest specific growth rate (μ=0.04 h^{-1}) (Fig. 2(a)). This tendency was also observed in the production of L-proline. However, Q_P and productivity showed a different tendency. They proportionally increased as a function of specific growth rate but decreased to above μ=0.08 h^{-1} (Fig. 2(b)). Thus the highest productivity of L-proline was 1.66 g L^{-1} h^{-1} at μ=0.08 h^{-1}. These results show that the production of L-proline in *C. acetoacidophilum* RYU3161 has the mixed growth-linked characteristics as shown in the different amino acid production [15,16].

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